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Inhibition of the Activation of Heat Shock Factor In Vivo and In Vitro by Flavonoids

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Transcriptional activation of human heat shock protein (HSP) genes by heat shock or other stresses is regulated by the activation of a heat shock factor (HSF). Activated HSF posttranslationally acquires DNA-binding ability. We previously reported that quercetin and some other flavonoids inhibited the induction of HSPs in HeLa and COLO 320DM cells, derived from a human colon cancer, at the level of mRNA accumulation. In this study, we examined the effects of quercetin on the induction of *HSP70* promoter-regulated chloramphenicol acetyltransferase (CAT) activity and on the binding of HSF to the heat shock element (HSE) by a gel mobility shift assay with extracts of COLO 320DM cells. Quercetin inhibited heat-induced CAT activity in COS-7 and COLO 320DM cells which were transfected with plasmids bearing the CAT gene under the control of the promoter region of the human *HSP70* gene. Treatment with quercetin inhibited the binding of HSF to the HSE in whole-cell extracts activated in vivo by heat shock and in cytoplasmic extracts activated in vitro by elevated temperature or by urea. The binding of HSF activated in vitro by Nonidet P-40 was not suppressed by the addition of quercetin. The formation of the HSF-HSE complex was not inhibited when quercetin was added only during the binding reaction of HSF to the HSE after in vitro heat activation. Quercetin thus interacts with HSF and inhibits the induction of HSPs after heat shock through inhibition of HSF activation.

Physiologic stress, including heat shock, enhances the synthesis of a limited number of intracellular proteins, the so-called heat shock proteins (HSPs) (19). The heat shock response has been observed in all cells so far tested, and some of the HSPs have been well conserved throughout evolution. In higher organisms, the induction of HSPs by heat shock or other stresses is regulated at the transcriptional and translational levels. The transcription of heat shock genes is regulated by the *cis*-acting heat shock element (HSE) in the promoter region and the *trans*-acting heat shock factor (HSF). The HSE consensus sequence was defined as the repeat of a 5-bp unit, NGAAN or NTTCN (2, 28), where N is any nucleotide, and the molecular cloning of HSF from yeast and *Drosophila* cells has been reported (5, 36, 38). In *Saccharomyces cerevisiae*, HSF is already bound to the HSE under normal conditions, and transcriptional activation is induced after heat shock at least partly through the phosphorylation of HSF, whereas in *Drosophila* and mammalian cells, HSF acquires DNA-binding ability only after heat shock through posttranslational modification of HSF (18, 34).

We have reported that quercetin and several other flavonoids inhibit the synthesis of HSPs, including HSP110, HSP90, HSP70, HSP47, HSP40, and HSP28, induced by heat shock, azetidine, or sodium arzenite treatment in two human cancer cell lines, HeLa and COLO 320DM cells (12). Quercetin inhibited the induction of HSP70 at the level of mRNA accumulation (12).

Flavonoids are a group of dyes commonly contained in higher plants (16). These compounds have many biochemical effects, such as inhibition of the enzymatic activities of ATPases (17), protein kinases (9), and adenylate cyclase (11). Binding of flavonoids to the type II binding sites of estrogen in murine uterine cells has been reported (20), an observation which suggests that flavonoids can bind specifically to some proteins in mammalian cells. Another bioflavonoid, luteolin, regulates the expression of the *nod* genes of *Rhizobium meliloti* in cooperation with *nodD* protein, a transcriptional regulatory factor of *Rhizobium* spp. (29).

In this study, we examined the effect of quercetin on the promoter region of the human *HSP70* gene, especially on the formation of the HSF-HSE complex after activation either in vivo by heat shock or in vitro by heat treatment, urea, or Nonidet P-40 (NP-40). Quercetin inhibited, both in vivo and in vitro, the binding of HSF to the HSE, as detected in a gel mobility shift assay. Although quercetin has also been reported to inhibit the activity of RNA polymerase II (24) and RNA polymerase from *Escherichia coli* (26), the inhibition of HSP70 synthesis was not due to the inhibition of RNA polymerase II activity under our experimental conditions in the RNase protection assay. The inhibition of HSP induction by quercetin was thus attributed to the inhibition of HSF activation through the interaction of quercetin with HSF.

MATERIALS AND METHODS

Cell culture and drug treatment. COLO 320DM cells, derived from a human colon cancer, were provided by the Japanese Cancer Research Resources Bank (32). Cells were cultured adhesively or in suspension in a 10-cm-diameter plastic dish containing Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum. Quercetin, flavone, and genistein were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO added to the medium was 0.25% (vol/vol). As a control, the

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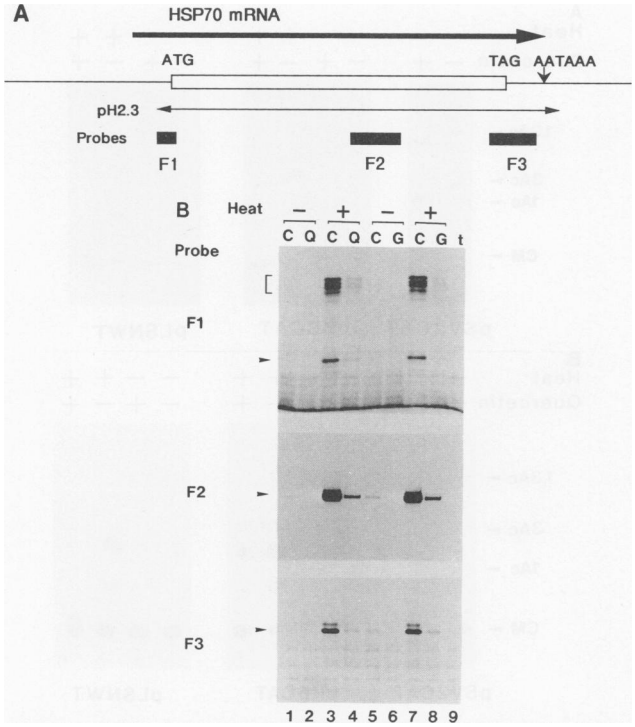

FIG. 1. Inhibition of accumulation of *HSP70* mRNA in the RNase protection assay. (A) Schematic diagram of the probes used for the RNase protection assay. F1 includes the initiation codon, and F2 lies approximately in the middle of *HSP70* mRNA. F3 contains the termination codon and a part of the 3′ untranslated region. (B) RNase protection assay with the three different probes shown in panel A. Test RNA and radiolabeled probes were prepared as described in Materials and Methods. The protected fragments are indicated by the bracket and arrowheads. Total RNA was prepared from non-heat-shocked (lanes 1, 2, 5, and 6) or heat-shocked (lanes 3, 4, 7, and 8) COLO 320DM cells preincubated for 6 h at 37°C with DMSO as a vehicle (C, lanes 1, 3, 5, and 7), 100 μM quercetin (Q, lanes 2 and 4), or 100 μM genistein (G, lanes 6 and 8). Yeast tRNA (1 μg) was hybridized with each probe instead of the test RNA and electrophoresed on lane 9 (t).

same concentration of DMSO was added. Quercetin and DMSO were purchased from Nacalai Tesque (Kyoto, Japan). Flavone and genistein were obtained from Extrasynthèse (Genay, France).

Preparation of RNA and RNase protection assay. Cells (2×10^6) were plated on a 10-cm-diameter plastic dish, and about 1×10^7 cells per dish were treated on the second day with 100 μM quercetin or genistein for 6 h. The cells were then either heat-shocked at 43°C for 90 min or kept at 37°C for the same period. Cells were harvested immediately after heat shock and lysed in a buffer containing 5 M guanidinium isothiocyanate, 5 mM sodium citrate (pH 7.0), 0.5% sodium *N*-lauroylsarcosine, and 0.1 M 2-mercaptoethanol (4). Total RNA was purified and stored in 70% ethanol. Three fragments excised from plasmid pH2.3, which encodes the human genomic *HSP70* gene (40), were inserted into pGEM-3Z (Fig. 1A), and antisense riboprobes were transcribed and uniformly labeled with T7 RNA polymerase and [α-^{32}P]CTP (800 Ci/mmol, 20 mCi/ml; Amersham Japan, Tokyo) according to the manual supplied by Promega Corporation (31). One microgram of total RNA was hybridized with approximately 3×10^6 cpm of each probe at 45°C for 14 h in 80% formamide buffer, containing 0.4 M NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4], and 1 mM EDTA (21). Unhybridized RNA was digested with RNase A (40 μg/ml) and RNase T_1 (2.8 μg/ml) and then incubated with proteinase K. The protected RNA probe was analyzed on a 6% polyacrylamide gel containing 7 M urea.

Transfection of plasmids and CAT assay. COS-7 or COLO 320DM cells were transfected by the DEAE-dextran method (3) with plasmids carrying the bacterial chloramphenicol acetyltransferase (CAT) gene as a reporter. pHBCAT contains the 2.4-kb upstream sequence of the human *HSP70* gene (41), and pLSNWT comprises the human *HSP70* promoter sequence to −188, including the HSE consensus sequence (39). Forty-eight hours after transfection, COS-7 cells were heat-shocked for 60 min at 43°C in the presence or absence of 50 μM quercetin, allowed to recover for 60 min at 37°C, and lysed to analyze the CAT activity on a thin-layer silica gel chromatograph by the standard procedure (10). In the case of COLO 320DM cells, transfected cells were divided into four dishes and heat-shocked for 90 min at 43°C with or without 100 μM quercetin after 42 h of transfection. pHBCAT and pLSNWT were kindly provided by R. I. Morimoto (Northwestern University, Chicago, Ill.).

Preparation of cell extracts for gel mobility shift assay. About 5×10^6 cells suspended in 10 ml of medium in a 10-cm-diameter noncoated plastic dish were treated with flavonoids for 6 h, heat-shocked at 43°C for 90 min, and then collected by centrifugation. Alternatively, about 1×10^7 cells were suspended in 1 ml of medium, transferred to a microcentrifuge, and heat-shocked, for the convenience of cell preparation. To prepare whole-cell extracts, cells were lysed in high-salt buffer by the method of Mosser et al. (23). Cytoplasmic extracts from unshocked COLO 320DM cells were prepared by the methods of Dignam et al. (6), with some modifications as described by Kimura et al. (14). In short, cells cultured in suspension in a Spinner flask were collected, kept on ice for 30 min in a hypotonic lysis buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 M phenylmethylsulfonyl fluoride, and 1 μg of leupeptin per ml, and then lysed by 40 strokes of a Dounce homogenizer with a loose pestle. The homogenate was centrifuged twice for 5 min at 5,000 × *g*, and the supernatant was ultracentrifuged for 1 h at 135,000 × *g*. The supernatant was dialyzed against a buffer containing 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Aliquots were stored at −80°C.

Gel mobility shift assay. In vitro activation of HSF by elevated temperature, urea, or NP-40 was determined with unshocked cytoplasmic extracts as reported by Mosser et al. (22). Quercetin was dissolved in DMSO and added to the cytoplasmic extracts at the indicated concentrations. The final concentration of DMSO added was 2.5% (vol/vol). Binding reactions with 10 μg of whole-cell extracts or 30 μg of cytoplasmic extracts were performed for 30 min at 25°C in 10 μl of binding buffer (20 mM HEPES [pH 7.9], 1 mM $MgCl_2$, 60 mM KCl, 12% glycerol, 1 mM dithiothreitol) containing about 0.1 ng of radiolabeled probe, 1 μg of poly(d[I-C]), and 0.25 μg of pUC19. Samples were electrophoresed on a nondenaturing 4% polyacrylamide gel, dried, and autoradiographed. The probe used to detect the DNA-binding activity of HSF was ^{32}P-end-labeled double-stranded HSE oligonucleotide encoding nucleotides −107 to −83 of the human *HSP70* gene (upper strand, GATCTCGGCTG GAATATTCCCGACCTGGCAGCCGA) (23). For the com-

petition binding experiments, HSE12, a mutant of the HSE oligonucleotide reported by Sorger et al. (upper strand, CTAGAGATCTCTAGAGATCTCTAGAGGATCCCCG) (35), and an octamer-binding site (*Sph*II-*Sph*I fragment of the simian virus 40 enhancer region, GAGAAGTATG CAAAGCATGCACTC) (25) as an unrelated DNA were used. The octamer-binding motif (described above) and SP1 and AP1 consensus oligonucleotides (upper strand of SP1, ATTCGATCGGGGCGGGGCGAGC; upper strand of AP1, TTCCGGCTGACTCATCAAGCG; purchased from Promega Corporation) were used to examine the effect of quercetin on other DNA-binding proteins.

RESULTS

RNase protection assay. Using Northern (RNA blot) analysis, we have reported that quercetin inhibited *HSP70* mRNA accumulation induced by heat shock (12). The same results were obtained with genistein and flavone (data not shown). β-Actin mRNA, used as an internal control, was not altered. An RNase protection assay with three different probes prepared from pH2.3 was performed, first, to analyze quantitatively the amount of *HSP70* mRNA inhibited by the drugs and, second, to detect short RNA fragments which might be present if the drugs act as inhibitors of RNA polymerase II.

The positions of the probes used for RNase protection assay are shown in Fig. 1A, designated F1, F2, and F3. F1 consisted of a 97-bp fragment including the initiation codon of the *HSP70* gene, and F2 covered a 225-bp region approximately in the middle of *HSP70* mRNA. F3 consisted of a 193-bp fragment including the termination codon.

Figure 1B shows the results of the RNase protection assay with each of the three fragments. The protected bands are indicated by a bracket and arrowheads. Quantitative estimation of the inhibitory effect of quercetin and genistein on *HSP70* mRNA accumulation is exemplified by the F2 fragment. The size of the protected band was, as expected, 225 nucleotides (nt). The F2 fragment of *HSP70* mRNA was markedly induced by a 90-min heat shock at 43°C (lanes 3 and 7), and this induction was inhibited, almost to the level seen without heat shock, when cells were treated with quercetin or genistein (compare lanes 4 and 1 or lanes 8 and 5). The constitutive low-level expression of the F2 fragment (lanes 1 and 5) was undetectable in cells treated for 7.5 h with quercetin (lane 2) or genistein (lane 6). No corresponding band was detected when the probe was hybridized with 1 μg of yeast tRNA (lane 9).

When the F1 fragment was used as a probe, four fragments were protected; three bands between 30 and 35 nt and one smaller band. Because the size of the bands protected by F1 was expected to be 97 nt, the sequence of the *HSP70* mRNA from COLO 320DM cells was considered to have either several point mutations or a polymorphism compared with the sequence near the 5′ terminus of pH2.3, which was prepared from the genomic DNA of human placenta (40). Similarly, the length of the bands protected by probe F3 was approximately 60 nt, which was smaller than the expected size of 193 nt (see Discussion). In either case, the quantitative changes in the protected bands after heat shock and/or drug addition were similar to the result obtained with the F2 fragment as a probe. Furthermore, the profile of the bands protected was similar with quercetin and genistein (compare lanes 1 through 4 with 5 through 8).

In experiments with these three fragments as probes, no extra bands that were partially protected by the probes were detected. The implications of these results will be discussed below.

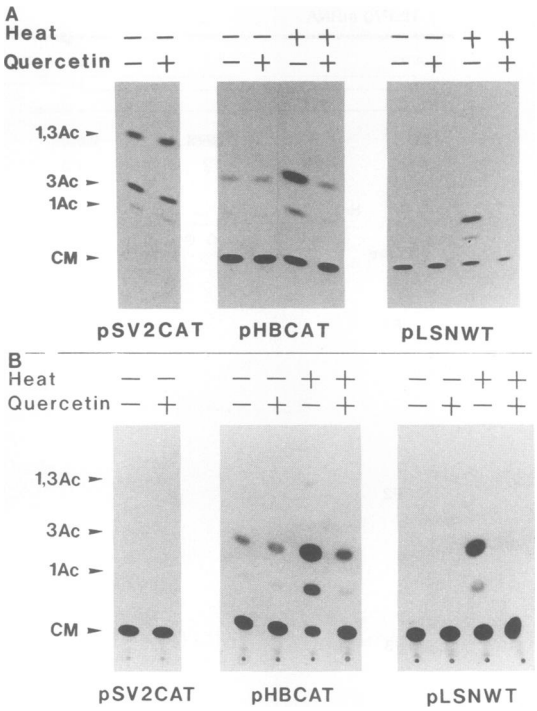

FIG. 2. Inhibition by quercetin of the CAT activity regulated by the promoter of the human *HSP70* gene. Cells were transfected with pSV2CAT, pHBCAT, or pLSNWT. pSV2CAT was used as a control. The bacterial CAT gene is regulated by 2.4 kb and 188 bp of the upstream sequence of the human *HSP70* gene in pHBCAT and pLSNWT, respectively. (A) After 48 h of transfection, COS-7 cells were heat-shocked at 43°C for 60 min in the presence or absence of 50 μM quercetin, allowed to recover at 37°C for 60 min, and then lysed to analyze the CAT activity as described in Materials and Methods. (B) Immediately after transfection of the plasmids, COLO 320DM cells were divided into four dishes and incubated for 42 h. Each dish was treated with or without heat shock (43°C, 90 min) in the presence or absence of 100 μM quercetin. CM, chloramphenicol; 1Ac, 1-acetate chloramphenicol; 3Ac, 3-acetate chloramphenicol; 1,3Ac, 1,3-diacetate chloramphenicol.

CAT assay. To examine whether the inhibitory effect of quercetin on the induction of HSPs after heat shock is due to its direct interaction with the promoter region of the human *HSP70* gene, pHBCAT or pLSNWT was transfected into the COS-7 or COLO 320DM cells, and CAT analysis was performed (Fig. 2). pHBCAT contains 2.4 kb of upstream sequence of the human *HSP70* gene, and pLSNWT has sequences up to −188 bp of the promoter, which includes both basal transcription units of CCAAT, the purine and TATA box, and the distal domain containing the HSE (39, 41). Both pHBCAT- and pLSNWT-transfected cells showed increased CAT activity after heat shock at 43°C. When quercetin was added to the medium during the heat shock and the following recovery period at 37°C, CAT activity in COS-7 cells was inhibited almost to the level seen without heat shock (Fig. 2A). When pSV2CAT (10) was transfected into COS-7 cells as a control, quercetin did not affect the CAT activity. To exclude the possibility that the transfection efficiency was different among the dishes, COLO 320DM cells were divided into four dishes after transfection, and the CAT activity was similarly analyzed. Quercetin also inhib-

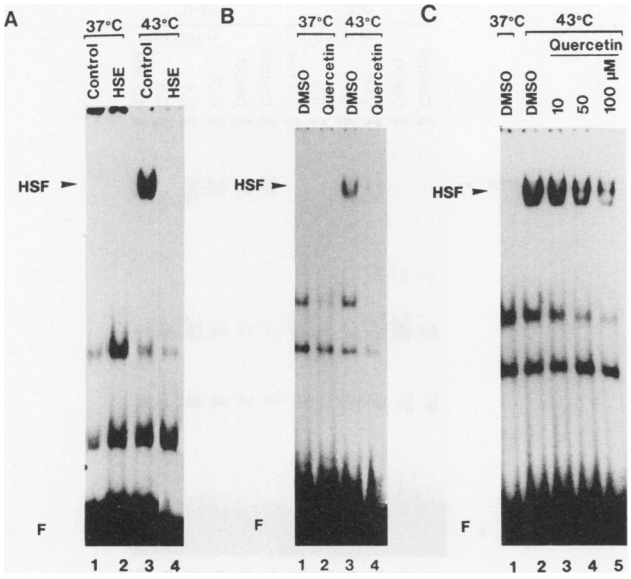

FIG. 3. Inhibition of HSF activation after heat shock in vivo in the presence of quercetin. The DNA-binding activity of HSF was examined by the gel mobility shift assay, with the end-labeled HSE oligonucleotide as a probe. (A) Whole-cell extracts were prepared from heat-shocked or non-heat-shocked COLO 320DM cells cultured in a 10-cm-diameter dish as described under Materials and Methods. To examine the sequence specificity of the binding of HSF, a 50-fold molar excess of nonradioactive HSE oligonucleotide was added (lanes 1 and 3, HSE). The arrowhead indicates the position of the HSF-HSE complex detected after heat shock (lane 3). F, free probe. (B) Vehicle (0.25% DMSO) or 100 μM quercetin was present in the medium for 6 h before heat shock. Cells were harvested immediately after a 90-min heat shock at 43°C, and the DNA-binding ability of HSF was subsequently examined. (C) Dose-dependent inhibition of HSF activation in vivo by heat shock with quercetin treatment. Cells were heat-shocked for 1 h at 43°C in a microcentrifuge in the presence or absence of quercetin. Quercetin was added to the medium at the following concentrations: lane 3, 10 μM; lane 4, 50 μM; lane 5, 100 μM. DMSO (0.25%) was added to the medium in lanes 1 and 2.

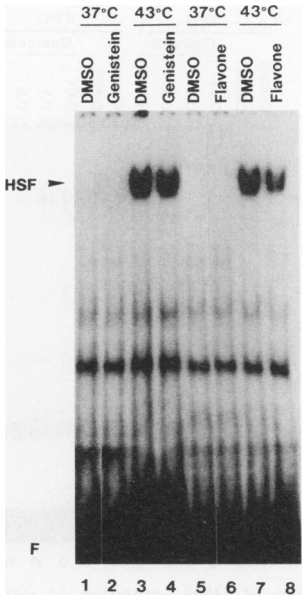

FIG. 4. Effect of genistein and flavone on HSF activation in vivo. About 10^7 cells were preincubated with 100 μM genistein or 150 μM flavone for 6 h prior to a 90-min heat shock at 43°C in a 10-cm-diameter dish. Whole-cell extracts were analyzed by the gel mobility shift assay. F, free probe.

ited CAT activity in COLO 320DM cells treated with heat shock (Fig. 2B). We could not detect CAT activity in pSV2CAT-transfected COLO 320DM cells.

HSF activation in vivo after heat shock. To examine the effect of quercetin on HSF activation in vivo after heat shock, we next performed the gel mobility shift assay with the end-labeled HSE oligonucleotide as a probe. Cells cultured in a 10-cm-diameter plastic dish were treated with 0.25% DMSO (vehicle) or 100 μM quercetin for 6 h prior to a 90-min heat shock at 43°C. Whole-cell extracts from heat-shocked and non-heat-shocked COLO 320DM cells were analyzed by the gel mobility shift assay. The HSF-HSE complex detected after heat shock (Fig. 3A, lane 3) disappeared when the assay was performed in the presence of a 50-fold molar excess of nonradioactive HSE oligonucleotide (lane 4). Quercetin inhibited the appearance of HSE-binding activity of HSF activated in vivo by heat shock (Fig. 3B). Two additional bands, which migrated faster than the HSF-HSE complex, were observed in whole-cell extracts, the density of which varied between experiments with no apparent regularity. These bands were considered to represent nonspecific bindings because they were not eliminated by the addition of excess unlabeled HSE oligonucleotides (Fig. 3A). The same result as that shown in Fig. 3B was obtained when cells cultured in suspension were collected in a microcentrifuge and heat-shocked at 43°C for 1 h with simultaneous addition of quercetin or DMSO to the medium (Fig. 3C). Dose-dependent inhibition of HSF activation by quercetin was observed (Fig. 3C, lanes 2 through 5). The HSF-HSE complex consisted of three adjacent bands, as was reported by Sorger and Pelham (35).

Figure 4 shows the effects of two other flavonoids on HSF activation in vivo. Genistein (100 μM) or flavone (150 μM) was added to the culture medium for 6 h prior to a 90-min heat shock at 43°C in a 10-cm plastic dish. Although these compounds inhibited the induction of HSPs at each concentration (12), genistein barely influenced the binding of HSF to HSE, and flavone moderately inhibited the specific binding of HSF with HSE.

HSF activation in vitro by heat treatment. Cytoplasmic extracts from nonshocked COLO 320DM cells were kept on ice (0°C) or at 43°C for 45 min in vitro, and the activation of HSF was detected by gel mobility shift analysis. The binding specificity was confirmed by competition experiments with nonradioactive HSE, HSE12 (a mutant HSE carrying six incomplete HSE consensus sequences), and the octamer-binding motif as competitive, mildly competitive, and noncompetitive oligonucleotides, respectively (data not shown).

We examined the effect of quercetin on in vitro activation of HSF by elevated temperature. The cytoplasmic extracts mixed with various concentrations of quercetin dissolved in DMSO were incubated for 45 min at 37 or 43°C. As a vehicle, DMSO was added at a final concentration of 2.5% (vol/vol). HSF activation was suppressed by the presence of 0.5 mM quercetin (Fig. 5, lane 11) and was totally inhibited by 1.0 mM quercetin (lane 12). Nonspecific binding was not affected by quercetin treatment at 37°C (see Fig. 8, lanes 1 to 6). At 43°C, two of the three nonspecific bands were slightly suppressed, whereas the lowest band was not affected by the presence of quercetin (Fig. 5, lanes 7 to 12).

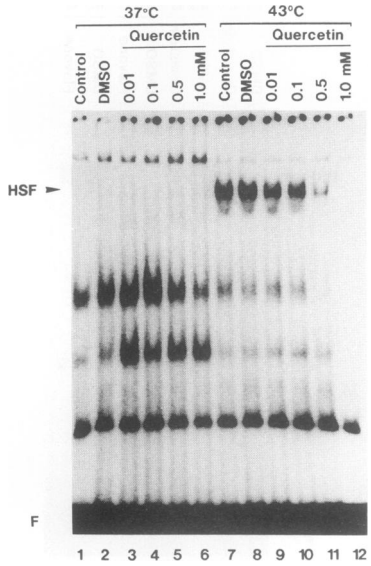

FIG. 5. Inhibition of HSF activation in vitro by heat in the presence of quercetin. Cytoplasmic extracts were incubated at 37°C (lanes 1 to 6) or 43°C (lanes 7 to 12) for 45 min in the presence or absence of various concentrations of quercetin. DMSO (2%) was added to the cytoplasmic extracts as a vehicle in lanes 2 and 8. To estimate the effect of DMSO, no vehicle was added in lanes 1 and 7 (control).

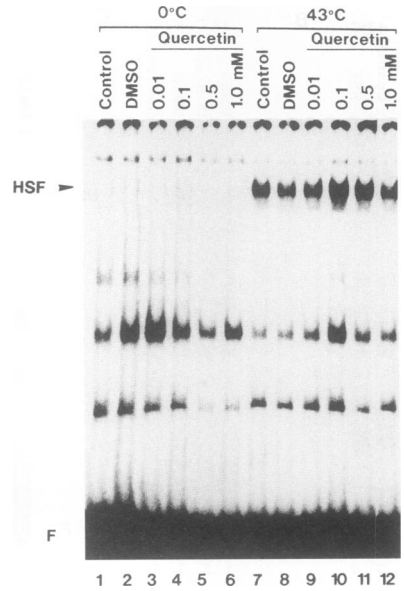

FIG. 6. Effect of quercetin treatment on the binding of activated HSF to HSE. The binding reaction was performed at pH 7.0 to avoid the possible conformational change of quercetin in an alkaline solution. Cytoplasmic extracts were kept on ice for 45 min (inactive form of HSF, lanes 1 to 6) or heated at 43°C for 45 min (activated form of HSF, lanes 7 to 12). These extracts were then mixed with various concentrations of quercetin or vehicle, and the binding reactions were carried out as described under Materials and Methods.

To elucidate whether the inhibition of formation of the HSF-HSE complex by quercetin in vitro was due to the repression of HSF activation itself or to inhibition of the binding of activated HSF to HSE, we added quercetin only when the binding reaction was performed. Quercetin dissolved in DMSO or DMSO alone as a vehicle was mixed with the binding buffer, and then cytoplasmic extracts which had been incubated for 45 min at 0 or 43°C in the absence of quercetin were added. The final concentration of DMSO added was 2% (vol/vol) in each experiment. In the alkaline binding buffer that we used (pH 7.9), quercetin was a brilliant yellow, reflecting some structural change. To negate the possibility that the ineffectiveness of quercetin in this experiment was due to a structural change, the reaction was performed in binding buffer at pH 7.0. The specific binding of HSF with HSE was not altered by the addition of quercetin up to a concentration of 1.0 mM (Fig. 6). Furthermore, quercetin did not affect the formation of the HSF-HSE complex in binding buffer at pH 7.9 (data not shown).

Interaction with other DNA-binding proteins. To investigate whether quercetin interacts with other DNA-binding proteins, we examined the effect of this compound on the binding of octamer-binding proteins (OBPs), SP1, and AP1. The promoter region of the human *HSP70* gene is reported to contain no octamer-binding motif. The specific binding of OBPs obtained in the whole-cell extracts of COLO 320DM cells to the octamer-binding motif was not affected by quercetin treatment in vivo (Fig. 7A, lanes 1 and 2). When cells were heat-shocked for 60 min at 43°C, the specific binding was slightly reduced, and quercetin did not alter the binding (Fig. 7A, lanes 3 and 4). Next, we incubated the cytoplasmic extracts with various concentrations of quercetin for 30 min at 37 or 43°C. The specific binding of OBPs to the probe was not affected significantly by incubation with up to 1 mM quercetin in vitro (Fig. 7B). The same experiments were performed with the SP1 and AP1 consensus sequences as probes. The specific binding of SP1 was not changed by the addition of quercetin in either in vivo or in vitro experiments (data not shown). The binding of AP1 was slightly reduced when cells were heat-shocked for 60 min at 43°C, as was observed with OBPs, and quercetin had no effect on the binding of AP1 (data not shown). We could not detect specific binding to the AP1 consensus sequence in the cytoplasmic extracts of COLO 320DM cells.

HSF activation in vitro by urea or NP-40. As was reported by Mosser et al. (22), HSF was activated by the incubation of cytoplasmic extracts with 1.5 M urea for 1 h at 37°C, and the binding of HSF to radiolabeled HSE was eliminated by the addition of a 50-fold molar excess of nonradiolabeled HSE (data not shown). Figure 8A shows the effect of quercetin on the in vitro activation of HSF by urea. Quercetin and urea were added to the cytoplasmic extracts simultaneously and then incubated for 1 h at 37°C. The formation of the HSF-HSE complex was inhibited by the addition of 0.5 or 1.0 mM quercetin. The same result was obtained with 2 M urea (data not shown).

HSF has also been reported to be activated in vitro by incubation of cytoplasmic extracts with nonionic detergents (22). We added quercetin to the cytoplasmic extracts during incubation with 0.2% NP-40 for 1 h at 37°C. Contrary to the result obtained after urea treatment, quercetin did not inhibit the HSF activation induced by 0.2% NP-40 in vitro (Fig. 8B). Similarly, quercetin had no effect on the HSF activation induced by 2% NP-40 (data not shown).

Kinetic studies of the effect of quercetin on HSF activation. We next examined the time course of the effect of quercetin on HSF activation both in vivo and in vitro. When COLO 320DM cells were heat-shocked for 5, 15, or 30 min at 43°C in a microcentrifuge, activated HSF was detected (Fig. 9A,

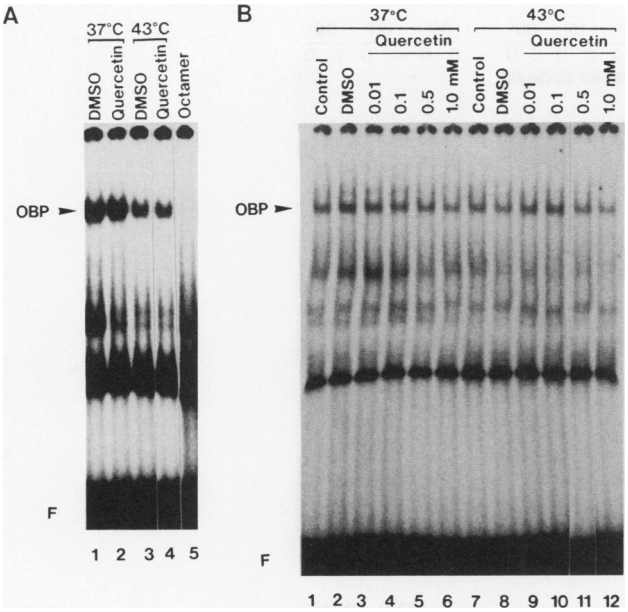

FIG. 7. Effect of quercetin treatment on the binding of OBPs to the octamer-binding motif. (A) Cells were transferred in a microcentrifuge and incubated at 37 or 43°C for 60 min in the presence or absence of quercetin. The DNA-binding ability of OBPs contained in the whole-cell extracts was analyzed by the gel mobility shift assay with the octamer-binding motif as a probe. (B) Cytoplasmic extracts of unshocked COLO 320DM cells were incubated at 37°C (lanes 1 to 6) or 43°C (lanes 7 to 12) for 30 min with or without various concentrations of quercetin. F, free probe.

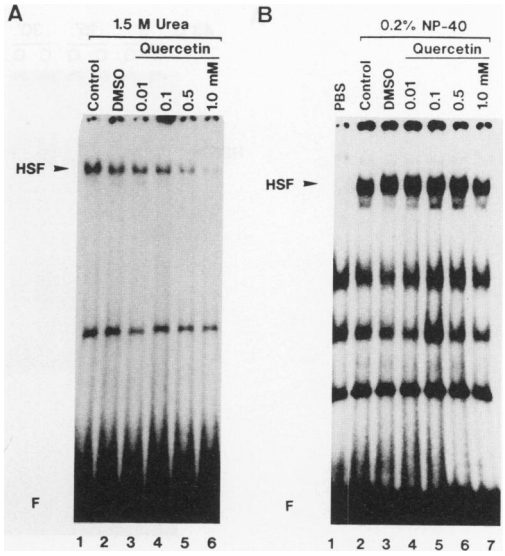

FIG. 8. Effect of quercetin on HSF activation in vitro by urea or NP-40. (A) Quercetin or DMSO was added to the cytoplasmic extracts at the indicated concentration simultaneously with a final concentration of 1.5 M urea and incubated for 1 h at 37°C. Neither DMSO nor quercetin was added in lane 1 (control). (B) Cytoplasmic extracts were incubated with 0.2% NP-40 for 1 h at 37°C, with or without quercetin at the indicated concentrations. PBS, phosphate-buffered saline control.

lanes 1, 3, and 5). Quercetin treatment for 6 h prior to heat shock reduced HSF activation (Fig. 9A, lanes 2, 4, and 6). However, when quercetin was added simultaneously with heat shock, the inhibition of HSF activation was not detected at 5 or 15 min after the temperature shift to 43°C (Fig. 9B, lanes 1 to 4). The inhibition was clearly observed after a 30-min heat shock and more intensively after a 90-min heat shock (Fig. 9B, lanes 5 to 12).

Figure 9C shows the kinetics of the effect of quercetin treatment on HSF activation in vitro by heat. Activated HSF was detected 5 min after the temperature shift, and quercetin inhibited its activation at each time point examined (Fig. 9C, lanes 8 to 12). HSF, once activated in vitro by a 30-min heat shock at 43°C (Fig. 9D, lane 2), was also reduced in its ability to bind to HSE when incubated with quercetin for a further 30 to 45 min at 43°C (Fig. 9D, lanes 3 to 6).

DISCUSSION

We previously reported that quercetin and several other flavonoids inhibited the synthesis of the HSPs induced by either heat shock, azetidine, or sodium arsenite treatment (12). This inhibition was exerted at the level of mRNA accumulation, as shown by Northern blot analysis with human genomic *HSP70* DNA (pH2.3) as a probe. Many kinds of stresses and conditions are known to induce the synthesis of HSPs, but inhibitors of HSP induction, other than deuterium oxide or glycerol, have not been described. These two agents are thought to protect the denaturation of thermolabile cellular proteins (8). The mechanism of inhibition of quercetin and other flavonoids is different from that of these compounds because flavonoids have not been reported to stabilize proteins, and furthermore, we have confirmed that quercetin, unlike glycerol or deuterium oxide (8), inhibited the induction of HSP synthesis at lower (42°C) or higher (45°C) heat shock temperatures, as revealed by Northern blot analysis or two-dimensional gel electrophoresis (13).

In this report, we showed that quercetin inhibited the activation of HSF. First of all, we performed an RNase protection assay with three different positional probes from a human genomic *HSP70* gene (pH2.3). The accumulation of *HSP70* mRNA, quantitatively estimated by the RNase protection assay in the presence or absence of quercetin or genistein, was well correlated with the amount of newly synthesized HSP70 protein, estimated by immunoprecipitation with specific antibody (12). Some flavonoids, including quercetin and flavone, have been reported to inhibit the activity of RNA polymerase II in permeabilized human fibroblasts and in vitro transcription with purified mouse RNA polymerase II (24). It has also been reported that in vitro transcription with RNA polymerase from *E. coli* is inhibited by several flavonoids, including quercetin (26). The effect of genistein on RNA polymerases has not been examined. If the flavonoids inhibited the synthesis of HSP70 induced by heat shock through the inhibition of RNA elongation, one would expect to detect shorter fragments than that protected by each probe in the RNase protection assay. Also, the ratio of the protected bands in the presence and absence of flavonoids will be expected to vary quantitatively among these three probes. However, such results were not observed, and we concluded that RNA polymerase II activity was not inhibited by quercetin or genistein under our experimental conditions in vivo. Moreover, quercetin did not affect the stability of the *HSP70* mRNA (13). Thus, quercetin was considered to inhibit the synthesis of HSP70 at the transcriptional level.

The several short fragments protected by the F1 fragment

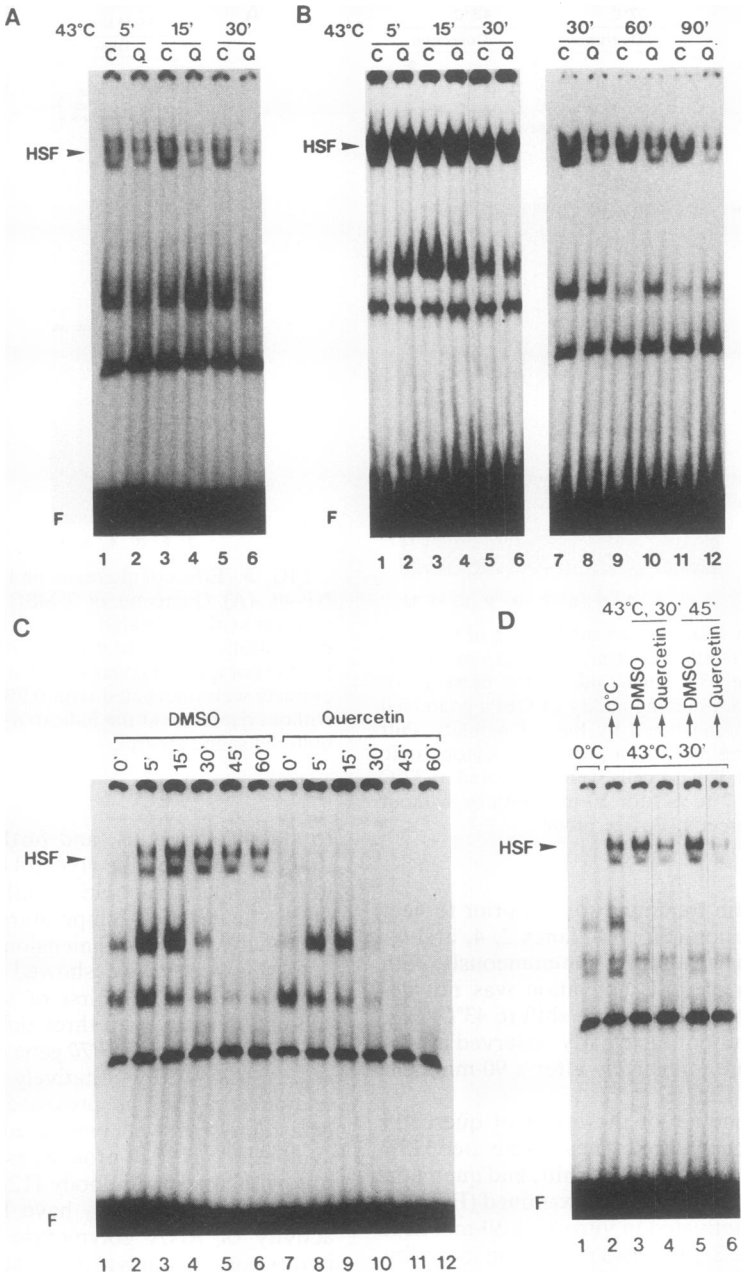

FIG. 9. Kinetics of the effect of quercetin on HSF activation in vivo and in vitro. The gel mobility shift assay was performed with whole-cell extracts (A and B) or cytoplasmic extracts (C and D). (A) COLO 320DM cells incubated for 6 h with DMSO (lanes C) or 100 μM quercetin (lanes Q) in a plastic dish were transferred to a microcentrifuge and heat-shocked at 43°C for the indicated periods (in minutes). (B) Aliquots of cells from a Spinner flask were suspended in 1 ml of medium containing 0.25% DMSO or 100 μM quercetin and heat-shocked in a microcentrifuge at 43°C for the indicated periods. Two independent experiments (lanes 1 to 6 and 7 to 12) were combined. (C) Cytoplasmic extracts from nonshocked COLO 320DM cells were incubated at 43°C for the indicated periods with DMSO (lanes 1 to 6) or 1 mM quercetin (lanes 7 to 12). (D) Cytoplasmic extracts treated at 43°C for 30 min (lanes 2 to 6) were incubated for another 30 min (lanes 3 and 4) or 45 min (lanes 5 and 6) after the addition of DMSO (lanes 3 and 5) or 1 mM quercetin (lanes 4 and 6). The activated form of HSF was detected after a 30-min heat shock at 43°C (lane 2). Lane 1, cytoplasmic extracts kept on ice.

probe were attributed to point mutations or to the polymorphism of COLO 320DM *HSP70* mRNA in the region covered by F1. Similar sequence divergences of several nucleotides have been reported for the two human genomic *HSP70* genes (7). The detection of two short protected RNA fragments in RNase protection assays has been described when a point mutation exists in the probe or in the test RNA (37). While a 193-nt band was expected in the RNase protection assay with the F3 fragment, only a 60-nt band was detected. This is probably due to the heterogeneity of the sequence of the 3′ untranslated region of COLO 320DM *HSP70* mRNA compared with that of pH2.3.

Quercetin inhibited the heat-induced CAT activity of COS-7 and COLO 320DM cells transfected with a plasmid

carrying the CAT gene downstream of the promoter region of the human *HSP70* gene. This inhibition was due to the blockade of the activation of HSF, as shown by the gel mobility shift assay, which used whole-cell extracts prepared from COLO 320DM cells heat-shocked in the presence or absence of quercetin.

With cytoplasmic extracts from unshocked cells, the activation of HSF has also been reported to be induced in vitro by elevated temperature (18), low pH, urea, detergents, or Ca^{2+} (22). In the present study, when the cytoplasmic extracts were treated by elevated temperature in the presence of quercetin, HSF activation was suppressed, as was the case in vivo. These results suggested that quercetin inhibited the activation of HSF, but the possibility remains that quercetin inhibited the binding of HSF to the HSE. To investigate the latter possibility, we heated the cytoplasmic extracts in the absence of quercetin and then added quercetin only during the binding reaction. With this procedure, quercetin was revealed not to affect the specific binding of activated HSF to the HSE. The concentration of quercetin required to inhibit HSF activation in vitro was fivefold higher than that required in vivo (100 μM in vivo versus 500 μM in vitro). It cannot be explained clearly now, because the actual concentration of quercetin accumulated in the cells is not known. One possibility is that higher concentrations of drugs are needed to access a specific protein like HSF in cytoplasmic extracts, in which various proteins are dissolved amorphously.

HSF activation in vitro by urea was also inhibited by the presence of quercetin. The observation that quercetin did not affect HSF activation by NP-40 in vitro may be attributed to the detergent action of NP-40, preventing the binding of quercetin to HSF. Mosser et al. (22) showed that the activation of HSF in vitro is inhibited by the presence of glycerol or deuterium oxide, compounds which stabilize labile proteins in vivo (8). Given the facts that quercetin and other flavonoids do not have protein-stabilizing activity and that *nodD* protein, a transcriptional regulatory factor of *Rhizobium* spp., modulates the expression of plant nodulation genes in combination with flavonoids (29), we suggest that quercetin interacts directly with HSF and inhibits its binding to the HSE, unlike deuterium oxide or glycerol.

It is of interest to examine the kinetics of quercetin treatment at the early stage of HSF activation. With whole-cell extracts, HSF activation in vivo was not inhibited in the first 5 to 15 min after heat shock when quercetin was added simultaneously. This might be due to a time lag needed for quercetin to enter the cells after the addition of the drug, because inhibition of HSF activation was already observed after a 5-min heat shock when the cells had been incubated with quercetin. Quercetin was shown by the gel shift analysis with cytoplasmic extracts to cause gradual inactivation of activated HSF as well as to inhibit HSF activation (Fig. 9D). We cannot clarify the mechanism of this inactivation because the active and inactive forms of HSF have not been established biochemically and the half-life of HSF is not known. At present, we can only postulate that quercetin might shift the equilibrium between the active and inactive forms of HSF to the inactive form both in vivo and in vitro.

In eukaryotes, the heat shock response is regulated by the interaction of HSF and HSE. In *S. cerevisiae*, HSF is already bound to the HSE under nonshock conditions and becomes transcriptionally active after heat shock (36). On the other hand, HSF binds to the HSE only after heat shock in human and *Drosophila* cells (18, 34). The activation of HSF in animal cells is reported to consist of two steps: acquisition of DNA-binding ability by the conformational change and transcriptional activation through the phosphorylation of HSF (18, 33). Our results suggest that quercetin inhibits HSF's acquisition of DNA-binding ability by affecting its conformational changes. The possibility that quercetin alters the half-life of HSF can only be evaluated once the biochemical properties of HSF have been more clearly defined.

As has been shown with flavone, some other flavonoids also have the ability to inhibit the activation of HSF in vivo. We did not examine the effect of flavone on HSF activation in vitro because of its low solubility in water. Although genistein inhibited the induction of HSPs, as revealed by Northern blot analysis and the RNase protection assay, it did not inhibit the activation of HSF in vivo. Since flavonoids have various biological effects both in vivo and in vitro (see the introduction), the discrepancy might be due to some other effects of genistein. Using permeabilized NIH 3T3 cells, Price and Calderwood reported that genistein inhibits transcriptional activation, but this drug did not affect the DNA-binding ability of HSF (30). It is of interest, as they discussed, that genistein inhibits the transcriptional activation of HSF through inhibition of the phosphorylation of HSF, since genistein is known to inhibit the activity of protein kinases (1, 27).

The regulation of the expression of HSF is very important not only in the stress response but also in physiological events, such as regulation of the cell cycle, differentiation, transformation, development, and so forth. Although many inducers of HSPs and HSF activation have been reported, the present study is the first to identify specific inhibitors for the activation of HSF. Inhibition of HSP expression has potential importance in the clinical aspect of hyperthermia, because tumors, through the induction of HSPs, acquire thermotolerance after repeated hyperthermia or even during a single hyperthermic treatment. Recently, we have found that quercetin inhibits the acquisition of thermotolerance in COLO 320DM cells (15). Thus, quercetin and other bioflavonoids are useful not only for establishing the positive and negative regulatory mechanisms for HSP expression but also for the clinical improvement of hyperthermic therapy of tumors.

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